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Biosynthesis of *N*-(α-Hydroxyethyl)lysergamide, a Metabolite of *Claviceps paspali* Stevens & Hall

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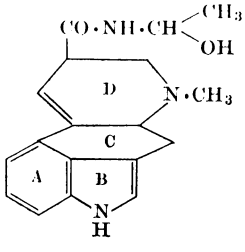
1. The biosynthetic origin of the amide substituent of *N*-(α-hydroxyethyl)lysergamide has been studied. 2. [1-^{14}C]Acetate, [^{14}C]formate, [2-^{14}C]mevalonic acid lactone, [2-^{14}C]indole, DL-[3-^{14}C]tryptophan, DL-[3-^{14}C]serine, DL-[2-^{14}C]alanine and [2-^{14}C]pyruvate were efficiently incorporated into the alkaloid, but not DL-[1-^{14}C]alanine or [1-^{14}C]pyruvate. 3. Only the DL-[2-^{14}C]alanine- and [2-^{14}C]pyruvate-derived alkaloid contained appreciable radioactivity in the amide substituent. 4. L-[^{15}N]Alanine-derived alkaloid was shown to be specifically labelled in the amide nitrogen. However, L-[^{14}C,^{15}N]alanine was found to be incorporated into the methylcarbinolamide substituent with an appreciable increase in the ^{15}N/^{14}C ratio, suggesting that alanine is not the direct precursor of this moiety.

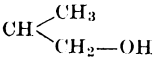
The development of a high-yielding strain of *Claviceps paspali* capable of producing *N*-(α-hydroxyethyl)lysergamide (I) when grown in submerged culture on a synthetic medium (Arcamone *et al.* 1961) provides a suitable system for biosynthetic studies of this alkaloid. Most investigations of ergot alkaloid biosynthesis have so far concerned the origin of the ergoline nucleus and this aspect of the *C. paspali* alkaloid (I) has already received some attention, the highly efficient incorporation of ^{14}C-labelled tryptophan having been demonstrated by Arcamone *et al.* (1962).

The structure (I), $C_{18}H_{21}N_3O_2$, was advanced primarily on the basis of its u.v. and i.r. spectra, which are characteristic of lysergamide derivatives, and also its ready cleavage under hot aqueous conditions, yielding D-lysergamide and acetaldehyde. Further evidence confirming this structure has been described by Arcamone, Camerino, Chain, Ferretti & Redaelli (1967), who obtained 8-ethylaminomethyl-6-methyl-$\Delta^{9,10}$-ergolene on reduction of the alkaloid with lithium aluminium hydride.

The present study is part of an investigation of the biosynthetic origin of the non-lysergic acid moieties of the ergot alkaloids, the C_2 methylcarbinolamide side chain of the alkaloid (I) providing the simplest known example of such a grouping.



(I)



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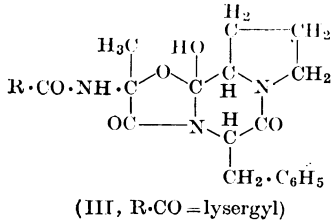

(III, R·CO = lysergyl)

MATERIALS AND METHODS

Chemicals. Chemicals used in preparing the culture medium were obtained from Fisons Scientific Apparatus Ltd., Loughborough, Leics., U.K. Mannitol and succinic acid were Standard Laboratory Reagent grade and KH_2PO_4, $MgSO_4,7H_2O$ and aq. NH_3 solution were Analytical Reagent grade.

Kieselgel G (E. Merck A.-G., Darmstadt, Germany) or Kieselgel N (Macherey, Nagel and Co., 516 Düren, Germany) were used to prepare thin-layer chromatograms.

Labelled compounds. DL-[2-^{14}C]Alanine was obtained from Nuclear Research Chemicals Inc., Orlando, Fla., U.S.A., and [2-^{14}C]indole from Centre d'Energie Atomique, Gif-sur-Yvette, France. All other radioactive compounds listed in Table 2 were purchased from The Radiochemical Centre, Amersham, Bucks., U.K.

L-[^{15}N]Alanine, 97 atoms % excess, was purchased

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from Schwarz BioResearch Inc., Orangeburg, N.Y., U.S.A.

Analytical procedures. (*a*) Total alkaloid. The total alkaloid content of culture filtrate samples was assayed by the colorimetric procedure of Voigt (1959).

(*b*) Thin-layer chromatography. A chromatographic system for the resolution of the alkaloid components was developed by using thin-layer silica-gel plates, heated for at least 1 h at 110°C, and a solvent mixture of chloroform–benzene–methanol (3:1:1, by vol.). The alkaloids were detected by their characteristic u.v. fluorescence and by the blue colour formed on spraying the chromatograms with 10% (w/v) *p*-dimethylaminobenzaldehyde in conc. HCl.

Approximate R_F values observed were: *N*-(α-hydroxyethyl)lysergamide and its C-8 epimer, 0.53 and 0.78 respectively; lysergamide and its C-8 epimer (isolysergamide), 0.37 and 0.71; ergometrine, 0.45. In this system ergotamine ran close to the solvent front whereas elymoclavine, agroclavine and chanoclavine remained near the origin.

(*c*) Fermentation procedures. The culture of *Claviceps paspali* Stevens & Hall used in these studies was developed from the original strain described by Arcamone *et al.* (1961) and was grown under similar conditions on a rotary shaker (200rev./min, 10cm eccentric throw) with 500ml Erlenmeyer shake flasks each containing 100ml of the following culture medium: mannitol, 50g; succinic acid, 40g; KH_2PO_4, 1g; $MgSO_4,7H_2O$, 0.3g; tap water, 1 litre adjusted to pH 5.2 with ammonia before autoclaving.

(*d*) Isotope assays. Radioactive isotopes were assayed in a liquid-scintillation spectrometer (Packard Tri-Carb model 3003) by using a modified Bray's scintillant (Bray, 1960).

Isotope ratios for samples containing ^{15}N were determined by the method of Rittenberg, Keston, Rosebury & Schoenheimer (1939) by using an Atlas M86 mass spectrometer.

High-resolution mass spectrometry was performed on an A.E.I. model MS9 mass spectrometer.

Addition of isotopically labelled precursors. All solutions of isotopically labelled precursors were sterilized by filtration through Millipore filters and added to the cultures under sterile conditions.

^{14}C-labelled precursors. Subject to a minimal total alkaloid content of 600μg/ml, on the ninth day (Table 1) precursor solutions (approx. 1ml) were added to two flasks and the cultures incubated for a further 24h before being harvested.

^{15}N-labelled precursors. Expt. 1. L-[U-^{14}C,^{15}N]Alanine (80mg in 4ml of water) was equally distributed between four flasks of a 9-day culture. Combined culture filtrate was collected after a further 24h. Expt. 2. A solution of L-[U-^{14}C,^{15}N]alanine (60mg in 3ml of water) was evenly distributed between three flasks of a 12-day culture and the cultures were incubated for a further 24h. Expt. 3. A portion (3ml) of a solution of L-[U-^{14}C,^{15}N]alanine (47.5mg in 10ml of water) was added to each of three flasks containing an 11-day culture and the cultures were incubated for a further 41h.

Isolation and purification of N-(α-*hydroxyethyl*)*lysergamide* (I). T.l.c. of a typical culture filtrate revealed the major alkaloid (I), lysergamide and their corresponding C-8 epimers, plus a fifth component, the R_F value of which was close to that of the major alkaloid. Its tentative chromatographic characterization as ergometrine (II) (mol.wt. 325) was supported by the presence in the mass spectrum of the chromatographically impure alkaloid (I) of a peak at *m/e* 325, which was absent from the spectrum of carefully purified alkaloid.

Table 1. *Production of* N-(α-*hydroxyethyl*)*lysergamide*

Typical alkaloid production results are given, showing colorimetric assay values at 9 days, and the yields of crude alkaloid recovered by chloroform extraction after incubation for a further 24h (vol. approx. 95ml/flask).

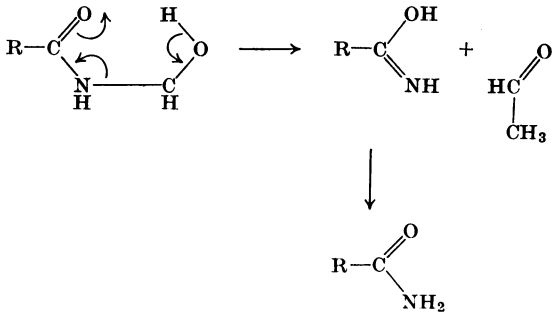
Expt. no.	Total alkaloid assay (μg/ml)	Recovered crude alkaloid (mg/flask)
1	1000	84.4
2	1100	89.4
3	700	62.1
4	1100	82.1
5	1900	175.0

The culture filtrate, adjusted to pH 7.2–7.5 with 2M-NH_3, was extracted with ice-cold chloroform (2vol.×3), which was then dried over anhydrous $MgSO_4$ and concentrated *in vacuo* below 20°C. The dried weight of the crude alkaloids was determined after drying in a vacuum desiccator over fresh P_2O_5 for 24h. Because of the light- and thermal instability of the alkaloids, these operations were carried out in a dimly lit cold-room.

Recovery of the alkaloid (I) was effected by dissolving the crude mixture in chloroform (approx. 100vol.) with gentle warming and, after filtration to remove a slight residue, partially evaporating under a stream of dry N_2 until crystallization commenced. The concentrate on refrigeration for 24h yielded crystalline alkaloid (I) corresponding to 30–50% of the initial crude alkaloid mixture (Table 1). The recrystallized alkaloid was usually contaminated with traces of ergometrine.

Alkaloid degradation procedures. The approach to the study of the biosynthesis of the carbinolamide side chain of structure (I) was based on the ready cleavage of the alkaloid into acetaldehyde (derived directly from the C_2 side chain) and lysergamide. The observed hygroscopic nature of the alkaloid, its ease of epimerization and the difficulty of separating it completely from both the ergometrine contaminant and the chloroform of crystallization complicated the accurate determination of the specific radioactivity of the radioactive alkaloid. It was therefore decided to determine the efficiency of incorporation of various precursors into the carbinolamide moiety by comparing the isotopic content of the acetaldehyde directly with that of the simultaneously formed lysergamide. The sum of the radioactivities of the easily purified lysergamide and acetaldehyde dimedone derivative provided an accurate measure of the radioactivity of the parent alkaloid.

The experimental conditions of alkaloid cleavage described by Arcamone *et al.* (1961) lead to epimerization at C-8, which not only decreases the yield of lysergamide but also complicates its purification. Investigation of alternative degradative procedures revealed that, on heating the alkaloid in xylene both acetaldehyde and lysergamide, free from isolysergamide, are obtained in



Scheme 1. Possible mechanism of pyrolysis of *N*-(α-hydroxyethyl)lysergamide. R = 6-methylergolin-9-en-8-yl.

high yield. A possible mechanism for this reaction is indicated in Scheme 1.

(*a*) Pyrolysis of *N*-(α-hydroxyethyl)lysergamide (I). In a typical pyrolysis experiment, *N*-(α-hydroxyethyl)lysergamide (107.3mg, 0.344mmol) in xylene (25ml) was heated at 150–160°C for 30min with rapid stirring in a slow stream of O_2-free N_2. The effluent gases were passed through two consecutive bubblers each containing an aqueous ethanolic solution of dimedone reagent (20ml), prepared by diluting a solution of dimedone (500mg) in ethanol (10ml) with water (30ml containing 4 drops of piperidine) and then filtering before use. The alkaloid dissolved after heating for 10min, and after a further 5–10min lysergamide began to crystallize from the pyrolysis solution. Acetaldehyde dimedone derivative also appeared in the bubblers at the same time and its formation was complete within 30min, when the effluent gas no longer formed a precipitate with Brady's reagent (0.32% 2,4-dinitrophenylhydrazine in 2M-HCl).

The lysergamide was filtered, washed with light petroleum (83.6mg, 0.31mmol, 91% yield) and recrystallized from acetone to give a chromatographically pure product, m.p. 242°C.

The acetaldehyde dimedone derivative was separated by filtration (83.4mg, 0.27mmol, 79% yield) and purified by crystallization from light petroleum (b.p. 60–80°C) to constant m.p. 137–138°C.

(*b*) Hydrolysis of lysergamide. In a typical experiment the lysergamide (80mg) was refluxed with M-NaOH (20ml) for 90min in a stream of N_2 and the resulting NH_3 was collected in a bubbler containing M-HCl (10ml). The reaction mixture was cooled to about 40°C, filtered and acidified to pH5.0 with M-H_2SO_4. When the solution was left overnight, lysergic acid hydrogen sulphate separated as colourless crystals. The yield averaged 30%, but with pure lysergamide (recrystallized four times) yields up to 60% were obtained.

RESULTS AND DISCUSSION

Incorporation of ^{14}C-*labelled precursors.* The results of experiments to determine the efficiency of incorporation of various ^{14}C-labelled precursors into the alkaloid and the distribution of ^{14}C label between the lysergic acid nucleus and the carbinolamide side chain are shown in Table 2.

The results confirmed the expected incorporation of tryptophan, acetate, mevalonic acid lactone and formate (Agurell, 1966) into the lysergic acid nucleus. [2-^{14}C]Indole was also utilized, but with a much lower efficiency than tryptophan, for which it can presumably serve as a precursor in this fungus. None of these substances appreciably labelled the carbinolamide side chain.

Sodium pyruvate, alanine and serine were selected on structural grounds as possible side-chain precursors. Although all three compounds gave rise to radioactive alkaloid, only alanine and sodium pyruvate labelled at C-2 or both C-2 and C-3 were efficiently incorporated into the C_2 side chain (approx. 40% of the total *N*-(α-hydroxyethyl)lysergamide radioactivity). With C-3-labelled serine as the precursor only 0.7% of the total radioactivity of the alkaloid was located in the carbinolamide side chain.

The relative incorporation values for ^{14}C-labelled alanine and pyruvate did not allow any decision to be made as to which is the more immediate precursor. The possibility of a direct condensation of lysergic acid, ammonia and pyruvic acid accompanied by decarboxylation is attractive in that it could account for the origin of the hydroxyl group present in the carbinolamide substituent and also the related moiety of the corresponding peptide alkaloids such as ergotamine (III). On the other hand the structure of ergometrine (II) suggests the direct utilization of alanine with subsequent reduction of the carboxyl group to a primary alcohol, and its coexistence with the methylcarbinolamide alkaloid (I) could be explained on the basis that alanine is a common biosynthetic intermediate.

Incorporation of [^{15}N]*alanine.* In an attempt to decide between alanine and pyruvate as the more immediate precursor of the side chain, incorporation of L-[^{15}N]alanine was investigated by using two independent procedures. Mass spectrometry was used in an attempt to study the distribution of ^{15}N in the labelled alkaloid without prior chemical degradation. Since the ion at *m/e* 267 corresponds to the lysergamide fragment, whereas that at *m/e* 223 is the same fragment after loss of the amide ($-CO\cdot NH_2$) group, the 268/267 and 224/223 *m/e* ratios would differ if the amide group is specifically enriched in ^{15}N relative to the two heterocyclic nitrogen atoms present in lysergamide. It was observed that the 268/267 *m/e* ratio of the L-[^{15}N]alanine-derived alkaloid showed an increase of 4.8% relative to that of the unlabelled alkaloid. The 224/223 *m/e* ratios of both samples, although very similar, showed some variation in successive runs. High-resolution analysis revealed that the

Table 2. *Incorporation of* ^{14}C-*labelled precursors into* N-(α-*hydroxyethyl*)*lysergamide* (*I*)

Precursors were added to 9-day shake cultures and the alkaloid was recovered after incubation for a further 24h. The methylcarbinolamide side-chain activity was determined by radioassay of the acetaldehyde dimedone derivative obtained by pyrolysis of the alkaloid.

Precursor	Radioactivity (μCi/flask)	Efficiency of incorporation into total alkaloid (%)	Radioactivity of alkaloid (I) in side chain (%)
L-[U-^{14}C]Alanine	15	3.7	41.3
L-[U-^{14}C]Alanine	25	1.7	39.1
DL-[2-^{14}C]Alanine	25	1.4	39.7
DL-[1-^{14}C]Alanine	15	0.31	—
DL-[1-^{14}C]Alanine	20	0.05	—
Sodium [2-^{14}C]pyruvate	15	1.6	38.9
Sodium [2-^{14}C]pyruvate	25	1.3	20.1
Sodium [2-^{14}C]pyruvate	20	3.2	37.0
Sodium [1-^{14}C]pyruvate	15	0.20	—
DL-[3-^{14}C]Serine	25	2.7	0.7
Sodium [1-^{14}C]acetate	25	10.1	<0.1
DL-[2-^{14}C]Mevalonic acid lactone	20	1.3	<0.1
DL-[3-^{14}C]Tryptophan	15	24.0	<0.1
DL-[3-^{14}C]Tryptophan	20	19.8	—
[2-^{14}C]Indole	20	5.1	<0.1
Sodium [^{14}C]formate	15	6.6	0.3

peak *m/e* 224 was a multiplet composed of the major peak *m/e* 224.0941 and a possible doublet at *m/e* 224.1261. The ion *m/e* 224.0941 ($C_{14}H_{12}N_2O^+$) was apparently the product of a retro-Diels–Alder fragmentation of ring D, with loss of $CH_2{:}N{\cdot}CH_3$, whereas the minor peak, *m/e* 224.1261, probably consisted of the two expected isotopic decarboxamidolysergamide ions, due to the ^{13}C and ^{15}N satellites, with theoretical *m/e* values of 224.1269 ($^{12}C_{14}{}^{13}CH_{15}N_2^+$) and 224.1206 ($C_{15}H_{15}{}^{14}N^{15}N^+$). The existence of isobaric peaks at *m/e* 224 explained the variation in 224/223 *m/e* ratios that precluded an accurate determination of the ^{15}N enrichment in the carbinolamide side chain.

The second approach utilized a chemical degradative procedure, which separated the amide and heterocyclic nitrogen atoms before ^{15}N assay. The ^{15}N-labelled alkaloid was diluted 3.5-fold with unlabelled alkaloid before pyrolysis to lysergamide, in which all three nitrogen atoms are retained. Determination of the ^{15}N content of the amide nitrogen of the lysergamide was effected by hydrolysis to ammonia, which was then oxidized to molecular nitrogen and assayed by mass spectrometry. The two heterocyclic nitrogen atoms of the lysergic acid were converted into ammonia by Kjeldahl degradation before ^{15}N assay as above. The amide nitrogen was found to contain 1.475 atoms % excess of ^{15}N (equivalent to 5.16% for the undiluted alkaloid), whereas the enrichment of the lysergic acid nitrogen atoms was negligible (less than 0.1 atom % excess of ^{15}N). This result shows clearly that the nitrogen from alanine, added to the culture on the ninth day of the fermentation, is utilized for biosynthesis of the carbinolamide side chain but not for the lysergic acid nucleus.

Incorporation of [^{14}C,^{15}N]*alanine*. The findings reported in the above two sections at first appeared to demonstrate the operation of a pathway for the biosynthesis of the methylcarbinolamide side chain directly from the nitrogen atom and C-2 and C-3 of alanine, requiring the oxidative loss of either the carboxyl group or the α-hydrogen atom as proposed by Castagnoli, Chain & Thomas (1966) and Gröger, Erge & Floss (1968). However, in three successive incorporation experiments using L-[U-^{14}C,^{15}N]alanine (Table 3) the ^{15}N/^{14}C ratio of the methylcarbinolamide substituent of the alkaloid was significantly higher than that of the alanine precursor.

This observation can be interpreted in at least two ways. (1) Alanine is the direct precursor of the methylcarbinolamide side chain, but its carbon skeleton and its amino group are incorporated with different efficiencies after equilibration with their respective endogenous carbon and nitrogen pools, which may have different sizes. (2) Alanine is not the direct precursor, but its carbon skeleton and amino group are differentially utilized after cleavage of the C–N bond and conversion into the obligatory intermediates. This possibility would include the mechanism involving pyruvate and ammonia suggested above. The apparent indirect utilization of L-alanine is consistent with the observations by Basmadjian, Floss, Gröger & Erge (1969) and by

Table 3. *Incorporation of* L-[U-^{14}C,^{15}N]*alanine into* N-(α-*hydroxyethyl*)*lysergamide*

For experimental details see the Materials and Methods section. The specific radioactivity of [^{14}C]alanine refers to C-2 and C-3 only and is equivalent to 66.6% of the total specific radioactivity. The value for the specific radioactivity of alanine used in Expt. 1 is derived from that supplied from The Radiochemical Centre. All results relating to the methylcarbinolamide side chain and the lysergic acid moiety have been corrected for dilution of the labelled material by unlabelled alkaloid.

Compound	Expt. no.	Specific radioactivity (^{14}C) (μCi/mmol)	Enrichment (^{15}N) (atoms % excess)	Ratio (^{15}N/^{14}C) (atoms % excess per μCi/mmol)	Side-chain ^{15}N/^{14}C ratio: Alanine	^{15}N/^{14}C ratio
Alanine	1	59.4	97	1.6	Expt. 1	1.5
	2	59.9	97	1.6		
	3	20.1	97	4.8		
Methylcarbinolamide side chain	1	2.13	5.16	2.4	Expt. 2	1.4
	2	0.95	2.19	2.3		
	3	0.16	1.96	12.0		
Lysergic acid	1	4.13	0.35	0.08	Expt. 3	2.5
	2	1.50	0.165	0.11		
	3	0.28	0.140	0.50		

S. Agurell (personal communication) that in *Claviceps paspali* D-lysergyl-L-[2-^{14}C]alanine is incorporated specifically into ergometrine (II) but not into *N*-(α-hydroxyethyl)lysergamide (I).

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